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CERTIFICATION REPORT

Certification of Plasmid DNA containing 356043 Soybean DNA Fragments

Certified Reference Materials ERM®-AD425

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Certification of Plasmid DNA containing 356043 Soybean DNA Fragments

Certified Reference Materials ERM®-AD425

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Disclaimer

Certain commercial equipment, instruments, and materials are identified in this report to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

ABSTRACT

This report describes the preparation, characterisation, stability and suitability studies of the certified reference material (CRM) ERM®-AD425 which contains a plasmid (pIRMM-0073) carrying a defined desoxyribonucleic acid (DNA) fragment specific for a genetic modification present in *Glycine max* (L.) Merill line 356043 as well as defined DNA fragment specific for the *Glycine max* taxon.

The CRM was processed in 2007 and certified in 2010 by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE. The CRM is available in plastic tubes containing 500 µL of the plasmid DNA in 1 mmol/L Tris, 0.01 mmol/L EDTA pH 8.0 buffer ($T_1E_{0.01}$) and 1 ng/µl ColE1 plasmid used as background DNA. Each tube contains approximately 10^9 copies of the ERM-AD425 plasmid which correspond approximately to 3.3 ng of DNA.

The plasmid contains a 99 bp fragment of the region that spans the 5' insertion locus of the event 356043 and a 259 bp fragment of the lectin gene (*le1*) specific for the soybean taxon. Both inserted fragments originated from DNA extracted from soybean 356043 seeds supplied by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

The certified values are the numbers of cloned DNA fragments for the 356043 and *le1* PCR targets per plasmid.

The recommended minimum sample intake is 50 µL to perform a dilution series. A minimum sample intake of 5 µL was used per real-time Polymerase Chain Reaction (PCR) assay.

	Number of DNA fragments per plasmid	
	Certified value [2)]	**Uncertainty** [3)]
Fragment of 5'-insertion-specific DNA / plasmid pIRMM-0073[1)]	1	negligible
Fragment of *le1* DNA / plasmid pIRMM-0073 [1)]	1	negligible

[1)] The sequence identity was confirmed by dye terminator cycle sequencing of the 5' insert-plant junction and le1 fragments present in in *Glycine max* (L.) Merill line 356043-5. The estimated error probability of the sequence identification of each fragment is lower than 0.0002 %.

[2)] The certified value is traceable to the International System of Units (SI).

[3)] The uncertainty is the standard uncertainty estimated by a type B evaluation based on the information described in chapter 8.1 of the certification report.

The intended use of this CRM is for calibration of the event-specific method for the quantification of the 356043 event validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), "Event-specific method for the quantitation of soybean line DP-356Ø43-1 using real-time PCR", accessible on the homepage of the EURL-GMFF (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) published on 11/02/2009.

GLOSSARY

35S	35S promoter, derived from cauliflower mosaic virus
6-FAM	6-carboxylfluorescein dye
6-VIC	Applied Biosystems proprietary dye
A	absorbance
AP(R)	ampicillin resistance
ANOVA	analysis of variance
BLASTN	Basic Local Alignment Search Tool for Nucleotides
bp	base pair
cp	copy number
EURL-GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed, formerly named Community Reference Laboratory (CRL GMFF)
CRM	Certified Reference Material
Ct-value	number of PCR cycles to pass a set threshold
df_{wb}	degrees of freedom within bottles
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
gDNA, gDNA$_\ell$, gDNA$_s$	genomic DNA, genomic DNA extracted from leaves, genomic DNA extracted from seeds
GMO	genetically modified organism
gm-hra	gene coding for a modified acetolactate synthase (ALS) enzyme which is not affected by the imidazolinone class of ALS inhibiting herbicides
*gat*4601	gene coding for glyphosate acetyltransferase (GAT) protein and conferring resistance to the herbicide glyphosate
le1	lectin gene from *Glycine max* (taxon-specific gene)
IQR	interquartile range
IRMM	Institute for Reference Materials and Measurements
LB	Luria-Bertani
M	molar mass
n	number of replicates
N	number of tubes analysed
N_A	Avogadro constant
N_d	number of data sets
N_{ds}	number of data sub-sets
NCBI	National Centre for Biotechnology Information
NIH	National Institutes of Health
OD	optical density
ORF	open reading frame
*ori*V	plasmid origin of replication
p	probability
PCR	polymerase chain reaction
pDNA	plasmid DNA
q	quality value assigned to a base-call in DNA sequencing
R^2	coefficient of determination
rel	relative
RNA	ribonucleic acid
RSD	relative standard deviation
S	size of a DNA fragment expressed in number of bp
s	standard deviation
SI	International System of Units
TAMRA	6-carboxytetramethylrhodamine
$T_1E_{0.01}$	1 mmol/L Tris, 0.01 mmol/L EDTA buffer, pH 8.0
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
TBE	Tris-borate-EDTA buffer
U_{CRM}	expanded uncertainty of certified value
u	standard uncertainty
u_{lts}	standard uncertainty contribution of the long-term stability of the material
UNG	uracil N-glycosylase
UV	ultra violet
$\overline{x}$	mean
x_t	DNA copy number ratio measured at the test temperature t
z	estimated error probability per base in DNA sequencing

Following international nomenclature the capital letter at the beginning refers to the protein, whereas lower case italic letters are used for the genes.

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1 INTRODUCTION

Legislation in the European Union demands the labelling of food products consisting of or containing "more than 0.9 % genetically modified organisms" (GMOs), provided the GMO has been placed on the market in accordance with Community legislation [1]. This enforces the necessity, on the one hand to develop and validate reliable quantitative measurement methods, and on the other hand to develop and produce reference materials to calibrate and control the correct application of detection methods. This threshold (0.9 %) is commonly understood as a mass fraction. In October 2004, the European Commission recommended expressing the content of GM food and feed as the percentage of GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes (EC) No 787/2004 [2].

Calibrants are required to determine the ratio of the number of copies of transgenic and taxon-specific genes. Purified plasmid DNA molecules (such as ERM-AD413) proved to be suitable calibrants for the quantification of gene copies [3, 4]. Here we describe the development of a new pure DNA calibrant containing a DNA sequence specific for the 356043 event and DNA fragments containing *Glycin max* taxon-specific sequences. As for any plasmid harbouring a single copy of a number of unique sequences, the ratio between the sequences is equal to 1.

The identity of the cloned sequences has been confirmed by the dye terminator cycle sequencing method. The nucleotide sequence of the 3' insert-plant junction cloned into the plasmid calibrant was identical to the nucleotide sequence provided by Pioneer Hi-Bred International, Inc.

Likewise, the nucleotide sequences of the *le1* fragment cloned into the plasmid calibrant were identical to the nucleotide sequences referred to as K00821.1 in the National Institutes of Health (NIH, Bethesda, MD, US) genetic sequence database (GenBank®) and correspond to the *le1* genes present in *Glycin max* species [5].

The soybean 356043 event is registered in the Organisation for Economic Co-operation and Development Unique Identification Registry (available via http://bch.cbd.int/database/lmo-registry/), according to the Regulation (EC) No 65/2004 of 14/01/2004 [6] establishing a system for the development and assignment of unique identifiers for GMOs. In accordance to this regulation, the soybean 356043 event received the unique identifier DP-356Ø43-5.

2 DESIGN OF THE PROJECT AND CERTIFICATION PROCEDURE

The major objective of the project was the production of a plasmid DNA reference material for the calibration of real-time PCR quantification methods.

The material is intended to be used as calibrant for the quantification of the 356043 event, expressed as the ratio between the number of copies of the 356043 event and the number of copies of a taxon-specific *Glycin max le1* gene. The plasmid calibrant is certified for containing one single DNA fragment of those two genes per plasmid. The cloned DNA fragments are identical to the sequences published in the NIH genetic sequence database (GenBank®).

The relative number of copies of the 356043 event per haploid genome of soybean that are present in a DNA extract can be calculated by application of the EURL-GMFF validated quantification method using the ERM-AD425 calibrant.

3 PARTICIPANTS

Processing and stability study

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* Laboratory holds accreditations ISO Guide 34 for the production of reference materials and ISO/IEC 17025 for DNA based GM measurements (accreditation body and registration number are mentioned).

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* Laboratory holds accreditation ISO/IEC 17025 for DNA based GM measurements (accreditation body and registration number are mentioned).

Certification

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE

* Laboratory holds accreditations ISO Guide 34 for the production of reference materials.

4 DESCRIPTION AND PROCESSING OF THE MATERIAL

4.1 DESCRIPTION OF THE STARTING MATERIAL

4.1.1 Cloning of the transgenic target

A 99 bp bp fragment containing part of the soybean chromosome and part of the 356043 transgenic sequence (5' insert-plant junction sequence) was amplified by the Platinum® Pfx DNA polymerase (Invitrogen, Carlsbad, CA, US) using as template genomic DNA extracted from seed powder of the 356043 event soybean. The amplicon The PCR product was purified using Qiagen PCR purification kit (QIAGEN Benelux B.V.,Venlo, NL) and ligated in the plasmid vector pCR2.1 (Invitrogen, Carlsbad, CA, US). *Escherichia coli* Top10 cells were then transformed with the ligation products. Transformed cells were selected on LB plates supplemented with 50 µg/mL kanamycin and 100 µg/mL ampicillin and identified by PCR screening using primers flanking the border regions of the cloned fragment. The correct insertion was confirmed by DNA sequence analysis on plasmid DNA isolated from single colonies. One single clone bearing the plasmid named pIRMM-0071 was selected.

4.1.2 Cloning of the endogenous targets

A 259 bp fragment lectin gene (*le1*) from soybean was amplified by the Platinum Pfx polymerase using the P-0712 and the P-0713 primers and genomic DNA extracted from 3560435 *Glycin max* as template (ANNEX 1). The amplicon was cloned in pCR2.1 (Invitrogen, Carlsbad, CA, US) to obtain the plasmid called pIRMM-0070. This plasmid was digested with *Eco*RI to release the fragment specific for the *le1* gene. Subsequently, this fragment was ligated into the *Eco*RI restricted pUC18 vector (pIRMM-0012) using the rapid DNA ligation kit (Roche, Mannheim, DE) to yield the plasmid pIRMM-0072 and *E. coli* Top10 cells were transformed with the ligation products. Transformed cells were selected on 100 µg/mL ampicillin-containing LB plates and identified by PCR screening using primers flanking the border regions of the cloned fragment. The correct insertion was confirmed by restriction enzymatic digestion using *Eco*RI on plasmid DNA isolated from single colonies.

4.1.3 Construction of the dual target plasmid

The plasmids pIRMM-0071 and pIRMM-0072 containing the 356043 and *le1* targets respectively, were both restricted with *Hin*dIII and *Pst*I, the combined digests were ligated and the ligation product transformed into *E. coli* TOP 10 competent cells. Transformed cells were plated on LB plates containing 100 µg/mL ampicillin and identified by restriction enzymatic digestion on extracted plasmid. The corresponding plasmid was named pIRMM-0073 (Figure 1).

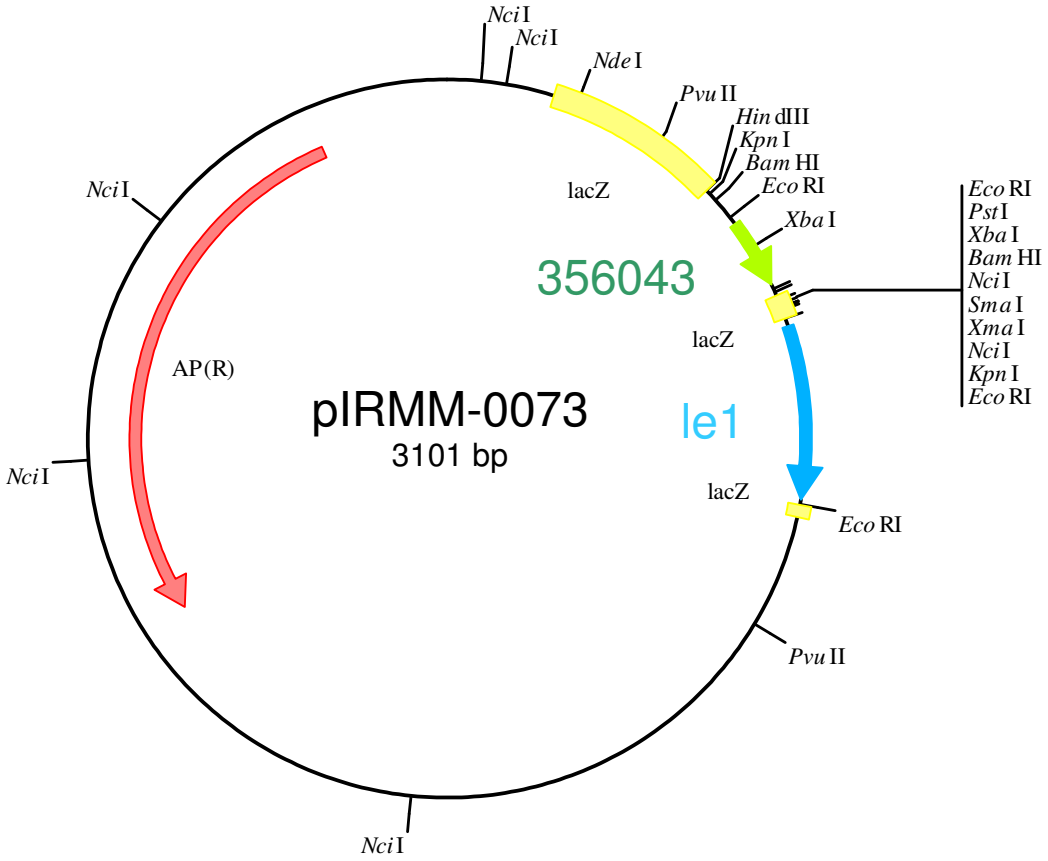

Figure 1: Circular map of pIRMM-0073 representing the 3' insertion-plant junction and the soybean endogenous target inserts as well as the enzyme restriction sites used in the cloning and restriction analysis

4.2 PURITY OF THE MATERIAL

The purity of the plasmid pIRMM-0073 was analysed by agarose gel electrophoresis. No other DNA bands than those expected after restriction of the pIRMM-0073 with *Eco*RI (Figure 2**,** lane 2), *Pvu*II (Figure 2, lane 3) and *Hin*dIII/*Pst*I (Figure 2, lane 4) could be seen after ethidium bromide staining. This confirmed the correct cloning of the fragments.

As no smear was visible after any of the restriction digests of the plasmid preparation and no RNA band was visible, it can be reasonably concluded that the plasmid preparation was not contaminated with external genomic DNA or a large amount of RNA molecules. However, traces of genomic DNA from host bacterial cell or traces of RNA molecules can not be excluded in the final plasmid preparation. Such traces do not influence the target sequence ratio.

Indeed a BLASTN 2.2.18+ analysis of the cloned target sequences did not reveal any nucleic acid sequences identity with bacterial genomic DNA sources from the NCBI databases (data not shown).

In addition, real-time PCR analyses of each target (356043 and *le1*) performed on either genomic DNA extracted from seed powder or plasmid DNA had comparable PCR efficiencies demonstrating no significant effect of potentially contaminating bacterial genomic DNA on the real-time PCR reaction.

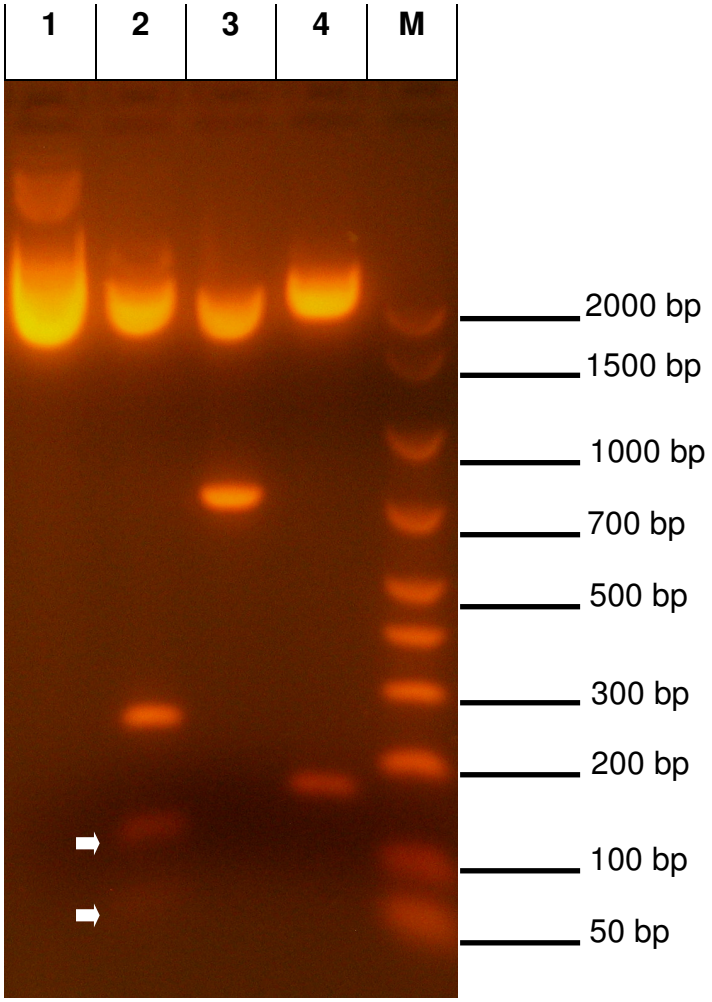

Figure 2: Restriction analysis of pIRMM-0073
Lane 1: pIRMM-0073 uncut; Lane 2: pIRMM-0073 restricted with *EcoR*I (expected fragments: 2683 bp, 259 bp, 115 bp, 44 bp [not visible on the gel, white horizontal arrows indicate the positions of smaller fragments]); Lane 3: pIRMM-0073 restricted with *Pvu*II (expected fragments: 2364 bp, 737 bp); Lane 4: pIRMM-0073 restricted with *Hind*III/*Pst*I (expected fragments: 2928 bp, 173 bp); M: molecular DNA marker (BioRad 50-2000 bp).

Such traces of *E. coli* genomic DNA or RNA may represent, however, a bias in the DNA quantification of the plasmid solution by UV and therefore an erroneous estimation of the number of plasmid copies in the tube. For that reason the DNA concentration in each tube can only be given as an approximate value.

Despite the fact that the enzyme restriction conditions were chosen to allow a full digestion of the intermediate plasmids used for the assembly of pIRMM-0073, it is very difficult to

prove that all plasmid populations were indeed fully digested, as traces of undigested plasmids will not be visible after gel electrophoresis and ethidium bromide staining.

The *E. coli* cells could be transformed with 3 populations of plasmids: pIRMM-0073 present in large amount and traces of both undigested pIRMM-0071 and pIRMM-0072. However, as those 3 synthetic plasmids have the same origin of replication (*oriV* from ColE1 plasmid) they belong to the same incompatibility group. As a result, the transformed bacterial clones can only bear one single plasmid. As the plasmid production was started from a single colony, only one type of plasmid can be present in a one colony. We can therefore conclude that each single bacterium extracted from one colony contains only one type of plasmid[1].

As additional proof of purity, plasmid DNA isolated from the transformed *E. coli* cells was sequenced completely to verify that all target DNAs were present and correctly cloned. The sequence analysis did not reveal the presence of a mixed population of plasmids.

Furthermore, digital PCR experiments have been carried out to determine the absolute number of *le1* and *356043* targets in ERM-AD425 following the same methodology as published earlier [8, 9]. Those measurements have confirmed the expected ratio between the two target sequences (DNA copy number ratio and its expanded uncertainty U, 1.03 ± 0.10, n = 5, k = 2). Based on all these observations, it can be reasonably concluded that no contamination with external genomic DNA or a relatively large amount of RNA molecules occurred during the plasmid preparation.

4.3 PROCESSING OF THE MATERIAL

Fifty mL of LB media containing 100 µg/mL of ampicillin were inoculated with a single colony of *E. coli* containing the plasmid pIRMM-0073 and shaken at 200 rpm overnight at 37 °C. The bacterial pellet was lysed in the presence of RNase, following the recommended protocol and buffers of a QIAprep Spin Miniprep kit (QIAGEN Benelux B.V., Venlo, NL). The plasmid DNA was purified on a silica membrane and eluted in 10 mmol/L Tris-HCl buffer, pH 8.5. The resulting DNA solution was stored at -20 °C until further use.

The plasmid DNA concentration and purity of the preparation were measured by UV spectrophotometry and fluorometry. A DNA mass concentration and standard deviation of 174 ± 7 µg/mL were obtained using the Picogreen® dsDNA quantitation kit (Molecular Probes Inc, Eugene, OR, US). The purity of the nucleic acid preparation was assessed spectrophotometrically by comparing the UV absorbance of the sample at 260 nm to that at 280 nm. Taking into account the generally accepted mean extinction coefficient for double-stranded DNA at 260 nm and 280 nm, pure nucleic acid samples are expected to have an A_{260}/A_{280} ratio approximately 1.8 and an A_{260}/A_{230} ratio above 2.0. The A_{260}/A_{280} and A_{260}/A_{230} ratios measured for the plasmid solution were 1.88 and 2.24 respectively, indicating a sufficient DNA purity; however, such values do not exclude traces of contaminating proteins [9].

[1] The synthetic vectors used (pUC18 and pCR®2.1) in our cloning strategy were chosen as being high copy vectors from the same incompatibility group (incQ). Those plasmids have their own origin of replication (oriV) and are able to replicate independently of the host chromosome. A bacterial host cannot however contain different plasmids that have the same mechanisms of replication, because the control of the replication (in Gram negative bacteria) is exercised through trans-acting molecules (theta replication mode). The inevitable consequence of this is that one of the plasmids would eventually be lost from the cell simply as a result of random partitioning of plasmids into daughter cells during cell division. Thus the plasmids would appear to be incompatible. Two or several plasmids from the same incompatibility group cannot coexist in the same cell [10].

The measured DNA mass concentration was used to calculate the number of copies of plasmid per volume. For this estimation, the following formulas were applied:

1 g of DNA contains x copies of a plasmid
with $x = N_A/[2 \cdot M \cdot S]$ (1)

where, N_A = Avogadro constant (mol^{-1}),
M = mean molar mass of a nucleotide (g/mol),
S = size of the plasmid molecule (number of nucleotides per plasmid DNA copy).

The number of plasmids per volume can be calculated by measuring the DNA concentration of the solution and using the following formula:

$$\text{copy number concentration [cp/µL]} = \frac{\text{DNA mass concentration [g/µL]} \cdot N_A\text{[bp/mol]}}{2 \cdot M\text{[g/mol]} \cdot S\text{[bp/cp]}} \quad (2)$$

A solution containing approximately 2×10^6 cp/µL of the plasmid pIRMM-0090 corresponding to a concentration of 6.6 pg/µL was prepared by diluting the stock plasmid solution in a background of ColE1 plasmid DNA (product number D9683, Sigma-Aldrich, Bornem, BE) that amounted to a final concentration of 1 ng/µL in 1.0 mmol/L Tris-HCl, 0.01 mM EDTA, pH 8.0 ($T_1E_{0.01}$).

4.4 FILLING OF TUBES

The above plasmid solution was sterilised by filtration through a nylon filter with a pore size of 0.2 µm and filled manually in pre-labelled high recovery polypropylene tubes with 500 µL of the plasmid solution under sterile conditions. Each tube contains approximately 10^9 copies of the ERM-AD425 plasmid, which corresponds to about 3.17 ng of DNA.

The tubes were placed in cardboard boxes each containing 100 tubes. Samples for the homogeneity, short- and long-term stability studies, and for additional characterisation were selected randomly from the entire batch. Each box was then sealed under light vacuum in a plastic pouch and frozen either at (-20 ± 5) °C or at (-70 ± 10) °C.

Each of the tube was identified by a numbered label as shown in Figure 3.

Figure 3: Prototype label for ERM-AD425

4.5 DISPATCHING OF TUBES

Tubes of the processed ERM-AD425 were dispatched to the participants in the interlaboratory comparison launched to evaluate the suitability of calibrants (Section 8.3) and obtain data used in the calculation of the certified value based on simplex real-time PCR measurements (Section 8.2). Unprocessed samples of plasmid DNA containing purified pIRMM-0073 in nuclease-free water were sent to two independent sequencing service companies to determine the complete nucleic acid sequence of the plasmid. All samples were packed in containers filled with dry ice.

5 PROCEDURES

5.1 METHODS USED FOR THE STABILITY STUDY

Simplex real-time PCR was used to identify and quantify the amount of both target DNAs present in the sample. The set up of the quantitative real-time PCR reaction was done according to the procedure described in the method validated by the EURL-GMFF for the event specific quantification of the soybean 356043 event [11]; the real-time PCR reaction volume used in this certification study was 25 µL; the baseline and threshold of the individual real-time PCR measurements were set automatically.

Briefly, the sequence identification and quantification of the 356043 transgenic target was performed using an event-specific method targeting a 99 bp fragment of the 5' plant junction region and a 74 bp fragment of the *le1* gene used as normaliser. The final forward and reverse primer amount of substance concentrations in the PCR reaction was 650 nmol/L and 750 nmol/L for the *le1* and the 356043 targets respectively, while the amount of substance concentration was 180 nmol/L and 200 nmol/L for the *le1* and 356043 probes, respectively [see also ANNEX 1 for the primer and probes sequences]. TaqMan® Universal PCR MasterMix analyses were carried out according to the manufacturer's instructions (Applied Biosystems, Lennik, BE) with thermal profile consisting of an initial denaturation step at 95 °C for 10 min, followed by 45 amplification cycles of 15 s at 95 °C and 1 min at 60 °C. The TaqMan® Universal PCR MasterMix contained AmpliTaq Gold® DNA polymerase for a better yield and a more robust 5' nuclease assay than AmpliTaq DNA polymerase and AmpErase® UNG to protect against subsequent reamplifications from PCR products minimising as such the carry-over contamination. The MasterMix also contained a passive reference dye to correct for inter-well signal variation caused by slight differences in the reaction volume.

Five µL of DNA solution were added to 20 µL master mix containing the appropriate primers and probe for the simplex real-time PCR. The 356043 probe was 5′ labelled with 6-VIC and the *le1* probe was 5′ labelled with 6-FAM. The fluorescence was recorded on-line (in real-time) during the PCR amplification process and its intensity was proportional to the number of target DNA sequences.

5.2 METHODS USED FOR CHARACTERISATION

5.2.1 Gel electrophoresis

The restriction pattern of plasmid DNA samples was analysed by gel electrophoresis, using a Sub-Cell GT agarose gel electrophoresis system (Bio-Rad Laboratories, Hercules, CA, US) and 2 % mass fraction agarose gel containing 0.5 µg/mL ethidium bromide. Ten µL aliquots of each enzymatic restriction mixture were electrophoresed at constant voltage (100 V) for 45 min in TBE buffer (90 mmol/L Tris-HCl, 90 mmol/L boric acid, 2 mmol/L EDTA, pH 8.0). DNA was visualised by UV light and photographed using a GeneGnome system (Syngene, Leusden, NL).

5.2.2 Fluorometry and UV spectrometry

The extracted plasmid DNA was quantified using the PicoGreen dsDNA Quantitation Kit according to the manufacturer's instructions using a lambda DNA standard solution. The purity of the DNA in the solution was analysed at 230 nm, 260 nm and 280 nm using a UV/VIS spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies Inc, Wilmington, DE, US). The ratios of the absorbances at 260 nm and 280 nm (A_{260}/A_{280}) and of the absorbances at 260 nm and 230 nm (A_{260}/A_{230}) were calculated to provide an estimation of the purity of the extracted DNA.

5.2.3 DNA sequencing

The plasmid pIRMM-0073 was fully sequenced by three independent laboratories at IRMM, at Eurofins Medigenomix GmbH and by the QIAGEN Sequencing Services.

Sequencing at IRMM was performed on an ABI Prism® 3130*xl* Genetic Analyser (Applied Biosystems, Lennik, BE) using the BigDye® Terminator v1.1 cycle sequencing kit protocol. Cycle sequencing utilises successive rounds of denaturation, annealing and extension in a thermocycler to create a linear amplification of extension products. With dye terminator labeling, each of the four dideoxy terminators is tagged with a different fluorescent dye. The 3130*xl* RapidSeq36_POP7 run module was used, and the samples were analysed using the ABI sequencing analysis software v5.2 Patch 2.

Eurofins Medigenomix GmbH (Martinsried, DE) performed the sequence by primer walking on both DNA strands. All sequences were generated using BigDye-terminator chemistry (version 3.1) of Applied Biosystems (Foster City, CA, US) following standard protocols. For PCR reactions Primus 96 HPL Thermalcyclers (MWG AG, Ebersberg, DE) were used. Sequencing reaction cleanup was done by gel filtration through Performa DTR 96 well plates (Edge Biosystems, Gaithersburg, MD, USA). Finally all reactions were run on ABI Prism® 3730*xl* capillary sequencers.

The sequence quality values which are a transformed estimate of the probability of correctness (1 - probability of error) are represented in a log scale from 0 to 50. The use of a log-transformed error probability facilitates working with error rates in the interval of highest interest (very close to 0). The quality value q assigned to a base-call is defined by the following equation:

$$q = -10 \log_{10} z$$

where z is the estimated error probability for a base.

The sequences generated by Eurofins Medigenomics had a quality value of at least 50, which means that the estimated probability for each base to be incorrect was less than 1/100 000.

QIAGEN Sequencing Services, was also performed on both DNA strands with the BigDye® Terminator v3.1 cycle sequencing kit according to the protocol supplied by Applied Biosystems on a Model 3700 and 3730 automated DNA sequencers (PE Biosystems). Assembly of all sequencing data was carried out using the STADEN software package (version 4.5; http://www.mrc-lmb.cam.ac.uk/pubseq) and the DNASTAR package (DNASTAR Inc., Madison, WI, US). The sequences provided by QIAGEN had an estimated probability to return an incorrect base estimated to be less than 1/20 000.

5.3 METHODS USED FOR ADDITIONAL CHARACTERISATION MEASUREMENTS

A study designed to perform the sequence identification and quantification of the targets *le1* and 356043 present in ERM-AD425 and for evaluation of the suitability of DNA calibrants used in the quantification of the 356043 event in soybean seed powder was included as part of the interlaboratory comparison organised to assess the copy number ratio of ERM®-BF425c [12]. The set up of the quantitative real-time PCR reaction was as described in Section 5.1.

For the purpose of the study comparing the behaviour of pDNA and gDNA three types of DNA were used: plasmid DNA ERM-AD425, genomic DNA extracted from seed powder

certified for its mass fraction content of 356043 (ERM-BF425c) and genomic DNA extracted from 356043 plant leaves. Genomic DNA from 356043 plant leaves (gDNA$_\ell$) was obtained at IRMM by DNA extraction of soybean leaves harvested from germinated 356043 soybean seeds homozygous for the GM event using the protocol for automated DNA extraction and the Chemagic DNA Plant Kit (Chemagen Biopolymer-Technologie AG, Baesweiler, DE) following the manufacturer's protocol. For the extraction of genomic DNA from seed powder (gDNA$_s$), samples of ERM-BF425c were used. The plasmid DNA, gDNA$_\ell$ extracted at IRMM and gDNA$_s$ were shipped to the participanting laboratories on dry ice. For each data set, the genomic DNA from seed powder was obtained by each participant using one of the three DNA extraction methods:

i) a modified cetyltrimethylammonium bromide (CTAB) method [13],
ii) CTAB (modified as mentioned above) followed by additional purification (Genomic-tip 20/G column kit and procedure from QIAGEN, Hilden, DE),
iii) DNA extraction method provided with the GENE*Spin* kit for DNA extraction (GeneScan Analytics GmbH, Freiburg, DE).

The extraction methods used in the study are described in detail in the report describing the certification of ERM-BF425c for its DNA copy number ratio [12].

6 HOMOGENEITY

6.1 Homogeneity assessment

The certified value for ERM-AD425 is expressed as number of DNA fragments per pIRMM-0073 plasmid. This quantity defines the structure (DNA sequence) of a pure substance (pIRMM-0073), therefore the homogeneity of the material is not considered in the estimation of the uncertainty of the certified value.

6.2 Minimum sample intake

In the frame of the short-term stability study and the interlaboratory comparison addressing the analytical behaviour of plasmid and genomic DNA with respect to the real-time PCR quantification of the 356043 event in soybean (Section 7.1 and 8.3), it was shown that using 50 µL ERM-AD425 solution for the preparation of a calibration curve by serial dilution led to reliable PCR results. The validity of the PCR measurements was, however, not tested with smaller starting volume intakes. Therefore, the recommended minimum sample intake for setting up a calibration curve for real-time PCR, using the described method [11], is 50 µL.

7 STABILITY

7.1 SHORT-TERM STABILITY STUDY

7.1.1 Design of the short-term stability study

The short term stability of ERM-AD425 was evaluated by analysing 5 tubes stored at (-20 ± 5) °C and (4 ± 3) °C for 1, 2 and 4 weeks. Three replicates from each tube were analysed (N = 5, n = 3) at several dilution levels. The same number of tubes (5) was stored at (-70 ± 10) °C as reference. Each reference tube was analysed in triplicate by simplex real-time PCR to reveal changes in the amount of the two fragments present in the plasmid due to the different test temperatures and times of storage.

7.1.2 Results of the short-term stability study

The ratio of both sequences was analysed and did not indicate any significant change at (4 ± 3) °C or (-20 ± 5) °C (Figure 4). DNA copy number ratios followed a normal distribution at all tested temperatures. When scrutinising the data, one outlier was detected by the single Grubbs-test at a 95 % confidence level. As no technical reason to exclude the data of one tube was found the outlying data was retained. Regression analysis was done for each of the storage temperatures to reveal any trend in the ratio between the two targets in relation to the time of storage. A t-test showed no significant change over the time period of 4 weeks (95 % confidence level) for the material kept at (-20 ± 5) °C and (4 ± 3) °C when testing the plasmids at final concentrations estimated to be between 400 000 cp/µL and 20 cp/µL.

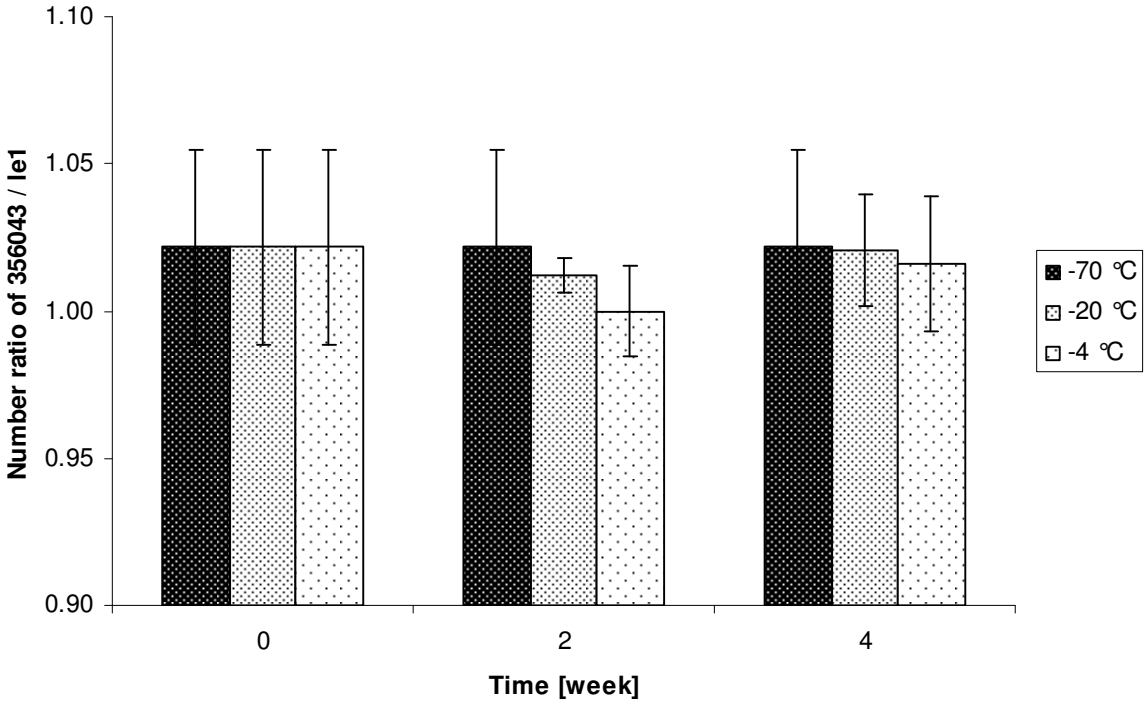

Figure 4: Short-term stability of ERM-AD425 stored at different temperatures for 0, 2 and 4 weeks and analysed by 356043 and *hmg* specific real-time PCR. The bars indicate the interval $\bar{x}$ ± standard deviation s for N = 5, n = 3.

7.1.3 Conclusion from the short-term stability study

The short-term stability on ERM-AD425 proved that it can be concluded that the ERM-AD425 withstands at least temperatures up to + 4 °C.

More information about the stability of materials has been gained on similar plasmid DNA that was processed using the same extraction and purification protocol, mixed in the same proportion with the same batch of ColE1 background DNA, based on the same vector, stabilised in the same buffer composition and stored in the same type of vials. Because of the similar processing and identical nature of the DNA, it is reasonable to think that both materials will have the same stability features.

Data from the isochronous long-term stability study of the ERM-AD413 stored at (18 ± 5) °C during 24 months (see details in Section 7.2) indicate that such materials can be shipped using cooling elements.

7.2 LONG-TERM STABILITY STUDY

7.2.1 Design of the long-term stability study

As mentioned above, ERM-AD425 has been produced following the same procedure as for ERM-AD413 [3] and is of the exact same nature. Therefore, to estimate the stability of ERM-AD425 during long-term storage, data obtained from the post-certification monitoring of ERM-AD413 for a period of 24 months was used. Tubes containing ERM-AD413 have been stored at (-20 ± 5) °C and (18 ± 5) °C to be analysed at those respective times (Figure 5).

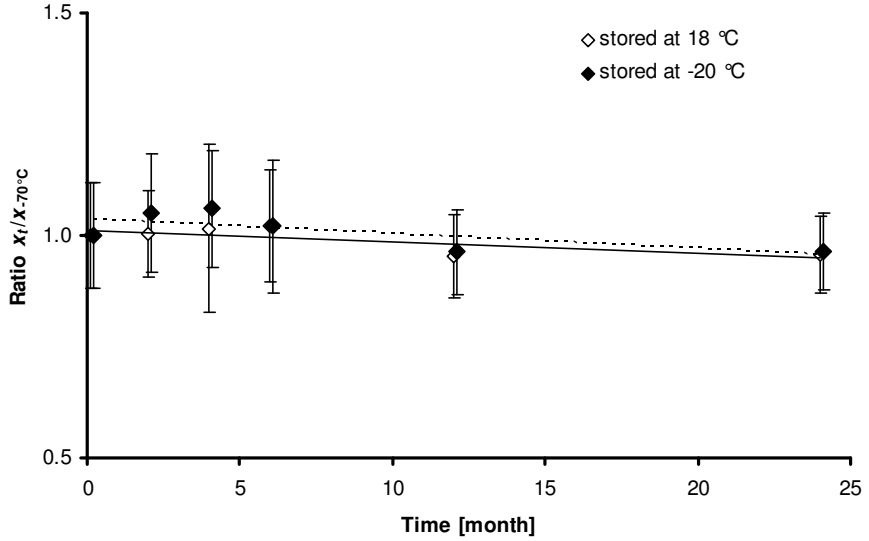

Figure 5: Long-term stability of plasmid DNA (ERM-AD413, MON810 calibrant) stored at (-20 ± 5) °C and (18 ± 5) °C for 24 months based on real-time PCR measurements. The stability is expressed as the ratio between the number ratio of MON810 and *le1* fragments per plasmid in samples stored at thr indicated temperatures and that in sample stored for the same period at the reference temperature [(-70 ± 10) °C], with the bars indicating the standard deviation. Each bullet corresponds to the mean mean ± standard deviation *s* of a minimum of 5 measurements. The dashed line is the regression line generated on the basis of the -20 °C data points, whereas the full line is the regression line generated on the basis of the (18 ± 5) °C data points.

7.2.2 Conclusions of the long-term stability study

Based on the stability study of ERM-AD413, a minimum shelf-life of 24 months at (-20 ± 5) °C can be ensured for real-time PCR applications. The ratio between the two inserted targets measured by real-time PCR remains unaffected, and the material can be, therefore, considered as suitable for use in real-time PCR measurements. The total amount of material present in the tubes does not decrease as the Ct values for the respective targets remains unchanged upon storage.

The long-term stability study does not show differences for the material storage at (-20 ± 5) °C or at (18 ± 5) °C in terms of copy number ratio. We recommend nevertheless to store the material at (-20 ± 5) °C, if the material needs to be stored for several months. Furthermore, yearly post-certification monitoring is planned to provide an additional control of the stability of ERM-AD425 to extend the shelf-life of ERM-AD425.

8 BATCH CHARACTERISATION

8.1 PLANNING

To verify the correct number ratio between the cloned DNA fragments in the ERM-AD425 calibrant, sequencing of the regions of DNA insertion in pIRMM-0073 was performed in-house on an ABI Prism® 3130*xl* Genetic Analyser (Applied Biosystems, Lennik, BE) using the BigDye® Terminator v1.1 Cycle Sequencing kit. Additionally, the full sequence of the plasmid was determined by two independent laboratories. The companies QIAGEN Sequencing Services (Hilden, DE) and Eurofins Medigenomix GmbH (Martinsried, DE) were asked to provide the full sequence of pIRMM-0073. The plasmid was sequenced on both strands by the two independent laboratories to insure a very high accuracy of the sequence generated and a full characterisation of the molecular composition of the plasmid DNA. The three sequences were compared to each other and submitted to GenBank® database (NIH) for BLASTN homology searches [14]. All three determined sequences were identical and confirming the correct cloning of the two fragments and the 100 % identity with published sequences. The ERM-AD425 plasmid could be accurately sized and consists of 3101 bp. The DNA sequencing also confirmed that each plasmid contained single *le1* and 356043 fragments. The uncertainty related to the sequence determination can be considered as negligible as the probability to report a wrong base was calculated to be less than 1/20000. As a clear consequence, the structurally defined ratio between the numbers of 3'-insertion-specific DNA and *le1* fragments in the plasmid pIRMM-0073 is 1.

The estimation of the DNA copy number ratio of the 356043 and *le1* detection fragments based on real-time PCR was done as part of the suitability study described in Section 8.3. The calibration curves prepared with the pDNA calibrant in the suitability study served as the source of real-time PCR measurement results to calculate the copy number ratio between the two targets.

8.2 ASSIGNMENT OF THE CERTIFIED VALUE

The material is certified to contain one 99 bp 5' insert-plant junction fragment and one 259 bp *le1* fragment per pIRMM-0073 plasmid[2] based on DNA sequencing. This certified value is based on the DNA sequence of pIRMM-0073 provided by three independent laboratories.

8.3 SUITABILITY STUDY

Thirty-two laboratories were selected on the basis of proven experience and quality management systems in place to investigate the comparability of the calibrant used for calibration of the real-time PCR measurements. PCR efficiencies as well as the linearity of the dilution series were calculated on the basis of serial dilutions of the plasmid calibrant (ERM-AD425) in $T_1E_{0.01}$ buffer, dilutions of gDNA extracted from leaves from germinated 356043 soybean seeds, labeled in the Figures and Tables as gDNAl, as well as dilutions of gDNA extracted from ERM-BF425c (containing 10.0 g/kg of 356043 soybean seed powder in non-GM maize powder) and labeled as gDNAs. ERM-BF425c is also certified for its copy number ratio [12].

To evaluate the comparability between the plasmid and genomic DNA, two parameters derived from the regression line generated by DNA serial dilution were compared and statistically analysed.

[2] The certified value expressed as number of DNA fragments per plasmid DNA molecule is an entitic number.

The PCR efficiencies as well as the linearity of the regression lines were calculated on the basis of serial dilutions in plasmid dilution buffer of the pDNA calibrant ERM-AD425, dilutions of gDNA from leaves as well as dilutions of gDNA extracted from the ERM-BF425c soya seed powder in nuclease-free water. To evaluate the behaviour of plasmid and genomic DNA, these two parameters have been compared and statistically analysed.

The first parameter was the PCR efficiency estimated for both transgenic and endogenous targets, using the three DNA types, *i.e.* pDNA ERM-AD425, gDNA extracted from leaves and gDNA extracted from ERM-BF425c seed powder. The PCR efficiencies were only compared if the respective data sets passed the selection criteria defined beforehand, namely the coefficient of determination (R^2) of the calibration curve, and PCR efficiency. These selection criteria were applied in order to avoid interferences of technically weak results generated by the participating laboratories [15]. Firstly, a R^2 value below 0.98 was not accepted within the study as it may reflect erroneous dilutions or inappropriate PCR amplification. Secondly, the PCR efficiencies interval was defined on the basis of pDNA ERM-AD425 and gDNA from leaves efficiencies (both materials used as calibrants in the study). The means of the PCR efficiencies were calculated for the endogeneous and transgenic targets using either the pDNA or gDNA from leaves calibrant. Four intervals were accordingly generated based on mean plus or minus one standard deviation. The minimum and maximum values of the resulting cut-off points were then used to define the lower and higher limits of the PCR efficiency of the study, *i.e.* 76 % and 110 % respectively. These values were consistent with the performance of the simplex real-time PCR method for quantification of the 356043 soya event observed with in-house performed studies and from results obtained by participants in the interlaboratory comparison.

Mean PCR efficiencies ranged from 89.7 % to 99.6 % (Table 1). For both targets, PCR efficiencies were highest using the pDNA calibrant and lowest for the gDNA extracted from leaves. Comparing the targets, PCR efficiencies were higher for the endogenous gene *lectin* than for the transgene 356043 (Table 1).

Single factor ANOVA analysis showed that efficiencies obtained by pDNA calibrant and gDNA calibrant are significantly different for the endogenous gene *lectin* ($p = 8.14 \cdot 10^{-10}$, 95 % confidence level, Figure 6) and the transgene 356043 ($p = 4.57 \cdot 10^{-8}$, 95 % confidence level, Figure 6).

The distribution of the PCR efficiencies and of their means for pDNA calibrant ERM-AD425, gDNA calibrant from leaves and gDNA extracted from ERM-BF425c seed powder were also compared and showed a large overlap for both targets for the three DNA types (Table 1, Figure 6). Statistical analysis proved that PCR efficiencies of pDNA, gDNA from leaves and gDNA from seed powder were significantly different for both *lectin* and 356043 target sequences.

A large number of outlier values were observed for the PCR efficiencies determined on the dilution series of the gDNA extracted from the ERM-BF425c seeds indicating some problems among the laboratories to work with a low number of DNA targets (Figure 6).

Table 1: Comparison of the real-time PCR efficiencies of gDNA extracted from leaves or seeds and pDNA; *n* indicates the number of data points, *s* is the standard deviation.

Target sequence	Mean PCR efficiency ± *s* [%]		
	ERM-AD425 pDNA	ERM-BF425c gDNA*s*	Leaves gDNA*ℓ*
lectin	99.6 ± 5.3 (N_{ds} = 65)	95.3 ± 5.5 (N_{ds} = 205)	92.6 ± 5.5 (N_{ds} = 79)
356043	95.7 ± 5.5 (N_{ds} = 73)	93.7 ± 6.5 (N_{ds} = 167)	89.7 ± 6.2 (N_{ds} = 56)

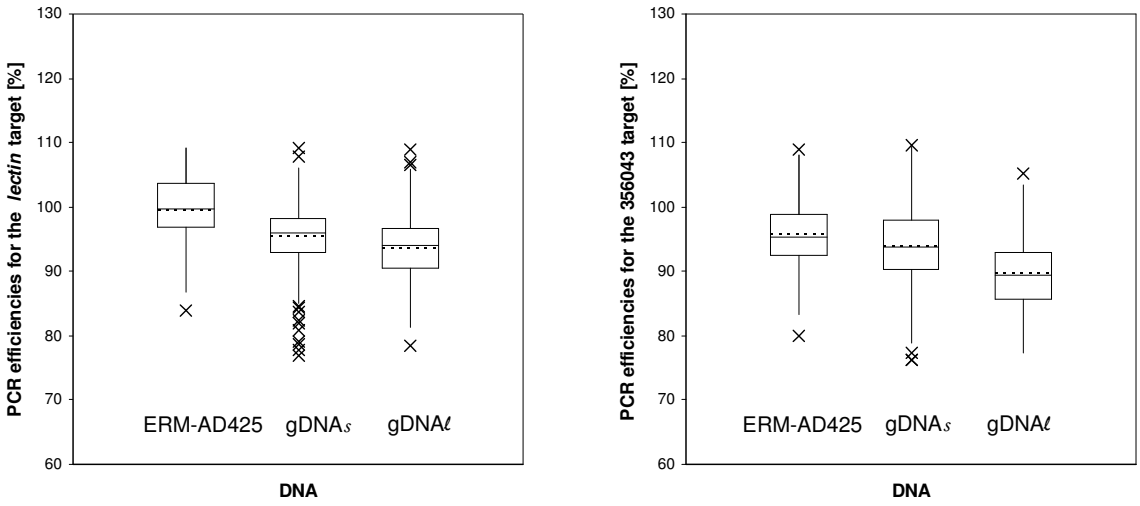

Figure 6: Box-and-whisker diagram illustrating the PCR efficiencies for the *lectin* and 356043 based on the dilution series performed using either ERM-AD425 calibrant or gDNA extracted from ERM-BF425c seed powder or from 356043 leaves are shown. The top and the bottom of the box are the 1st and 3rd quartiles, respectively; the full line near the middle of the box is the median. The ends of the whiskers are determined by substracting 1.5 times the interquartile range (IQR) from the 1st quartile, and adding 1.5 times the IQR to the 3rd quartile, respectively. The dashed line corresponds to the mean of each group, whereas extreme values outside the 95 % confidence level are marked with x.

The second parameter studied was the coefficient of determination (R^2) that provides information about the fitting of data to a linear regression line obtained for both targets and the three types of DNA (Table 2, Figure 7).

No major differences were observed between the three types of DNA for both targets. However, R^2 values were slightly higher for the calibrants than for the gDNA extracted from seed powder, revealing again the difficulties encountered by the laboratories to correctly quantify low number of DNA targets in the diluted samples (Table 2, Figure 7).

Table 2: Comparison of the coefficient of determination (R^2) of pDNA (ERM-AD425) and gDNA extracted from leaves or seeds; N_{sd} is the number of data subsets, s is the standard deviation.

Target sequence	Mean $R^2 \pm s$		
	ERM-AD425 pDNA	ERM-BF425c gDNA$_s$	Leaves gDNA$_\ell$
lectin	0.998 ± 0.002 (N_{ds} = 65)	0.997 ± 0.004 (N_{ds} = 205)	0.998 ± 0.003 (N_{ds} = 79)
356043	0.995 ± 0.003 (N_{ds} = 73)	0.994 ± 0.005 (N_{ds} = 167)	0.997 ± 0.002 (N_{ds} = 56)

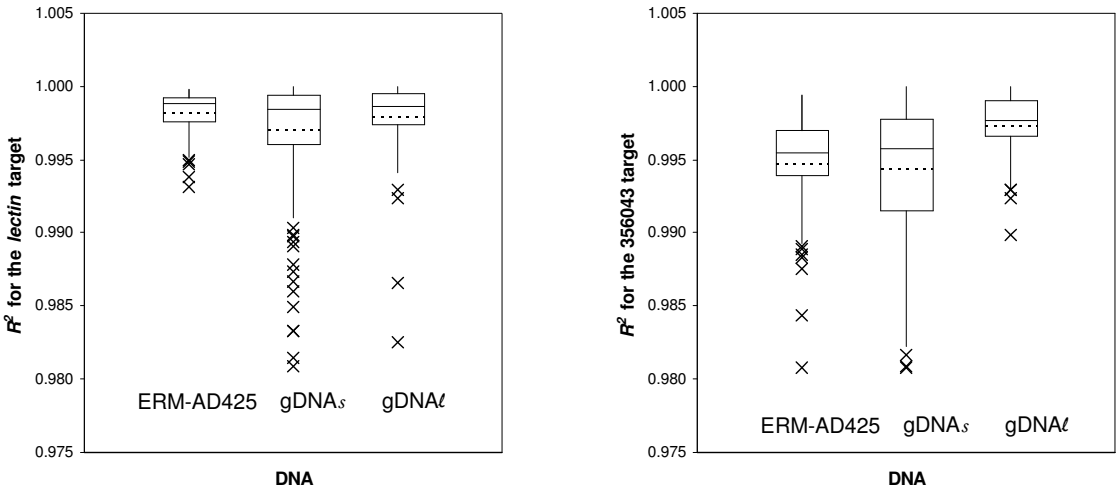

Figure 7: Box-and-whisker diagram representing the coefficient of determination (R^2) for the *lectin* and 356043 target genes based on the dilution series performed using either ERM-AD425 calibrant, gDNA extracted from ERM-BF425c seed powder or from leaves are shown. A description of a box-and-whisker diagram is given in Figure 6.

Finally, the 356043 DNA copy number ratio of the ERM-BF425c has been compared using either pDNA ERM-AD425 or the gDNA$_\ell$ as calibrants (Figure 8, Table 3). For this study, copy number ratios obtained per day from accepted data sets were compared. The data sets from both calibrants follow a normal distribution. Though the DNA copy number ratios obtained for ERM-BF425c by the two different calibrants overlap, the mean DNA copy number ratios are very different. Single factor ANOVA analysis confirms that the data sets calibrated with ERM-AD425 are significantly different from the data sets calibrated with gDNA$_\ell$ ($p = 1.36 \cdot 10^{-9}$, 95 % confidence level) (Figure 8). Therefore the DNA copy number ratios obtained when calibrated with ERM-AD425 or gDNA$_\ell$ cannot be pooled.

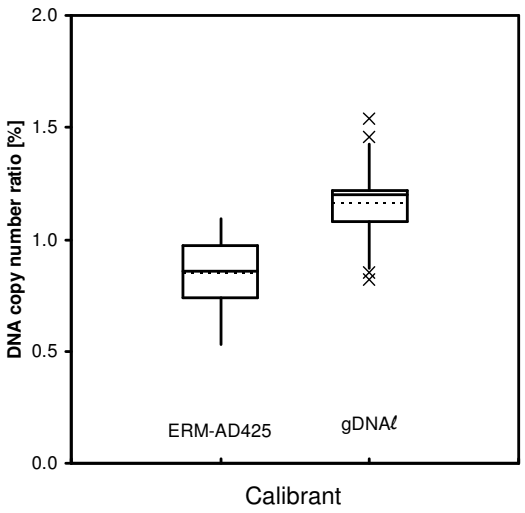

Figure 8: Box-and-whisker diagram and distribution graph of 356043 copy number ratios in ERM-BF425c using either pDNA ERM-AD425 or gDNA extracted from 356043 leaves as calibrant. A description of a box-and-whisker diagram is given in Figure 6.

Table 3: GM content in ERM-BF425c expressed in copy number ratio and calibrated with either ERM-AD425 or gDNAℓ extracted from 98140 leaves; N_{ds} is the number of accepted data sub-sets, *s* is the standard deviation.

	DNA copy number ratio ± *s* [%]	
	pDNA	gDNAℓ
ERM-BF425c	0.85 ± 0.14 (N_{ds} = 30)	1.15 ± 0.18 (N_{ds} = 26)

In the study reported here, statistical analyses have shown that the two calibrants, *i.e.* pDNA ERM-AD425 and gDNAℓ from leaves behave in a different way with respect to the PCR efficiencies of the transgenic and endogenous target sequences (Figure 6). The individual PCR efficiency of each target sequence has, however, a significant impact on GM quantification by real-time PCR. Therefore, the effect of a low difference in PCR efficiencies of the transgenic and endogenous targets on GM quantification by real-time PCR can generate a large difference in copy number due to the exponential nature of the PCR amplification. Consequently, such significant differences in PCR efficiencies of the calibrants may explain the difference between the mean values (Table 3).

An additional measurement of gDNA*s* extracted from ERM-BF425c was done by digital PCR in order to identify the true value. Digital PCR is a real-time PCR method that is independent of a calibrant but dependent on the same PCR chemistry, primers and probes as real-time PCR. Digital PCR involves distributing the PCR solution containing template nucleic acid molecules across a very large number of individual partitions prior to amplification. Following PCR amplification, a count of the proportion of partitions containing a detectable number of PCR amplicons can be used to estimate the total number of template DNA copies in the original DNA extract. The DNA copy number ratio was then calculated from the total number of template DNA copies. The DNA copy number ratio and its expanded uncertainty (k = 2) obtained by simplex digital PCR for gDNA extracted from ERM-BF425c was 1.02 ± 0.04 % (N = 3, n = 21, with each n measured in 5 replicate measurements) and is in line with the theoretical copy number ratio of 1 for ERM-BF425c.

Based on this study it can be concluded that both calibrants, *i.e.* pDNA ERM-AD425 and gDNA extracted from soya 356043 leaves are suitable to calibrate quantitative PCR method applied here. However, the user should be aware that the choice of calibrant influences the measured copy number ratio and can lead to significant different results. Using the pDNA calibrant ERM-AD425 the measured GM content might be underestimated by about 15 %. On the other hand, when gDNA extracted from leaves is used as a calibrant the GM content might be overestimated by 15 %. Studies have indicated that the most suitable approach is to set a reference system based on pDNA as described in the certification report [3] as the primary calibrant for DNA copy number ratio measurements together with the approved GM quantification method published by the EURL-GMFF [11].
As shown in Figure 9, ERM-AD425 can be diluted down to 1/100 000 in $T_1E_{0.01}$ buffer, and can be easily quantified for both targets.

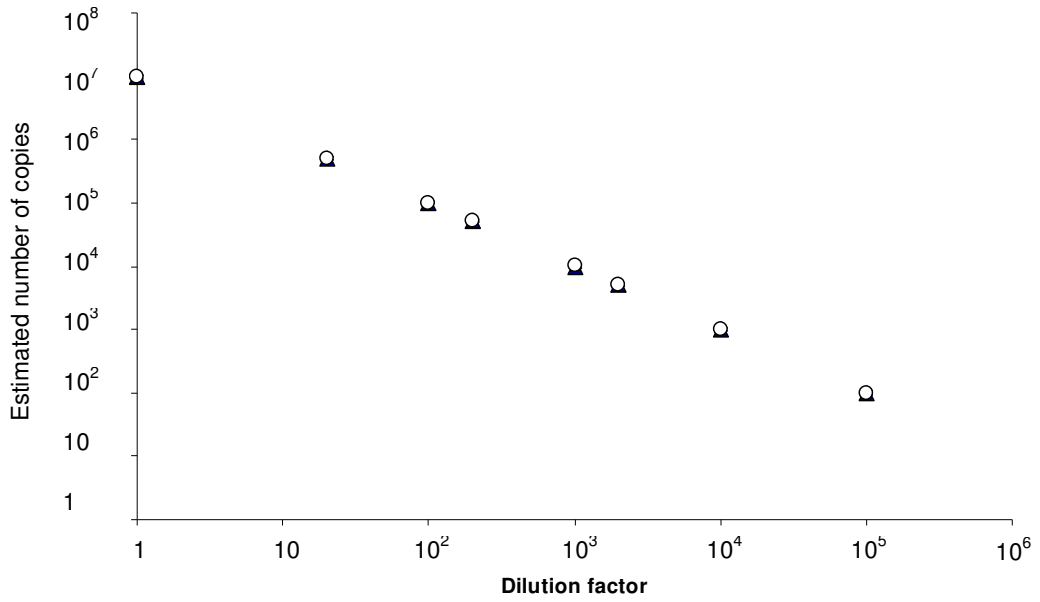

Figure 9: Comparison of estimated numbers of copies of the *lectin* (○) and 356043 (▲) target sequences by simplex real-time PCR methods applied for a serial dilution of ERM-AD425 ($N = 20$) in T1E0.01 buffer. The copy numbers were estimated using the PicoGreen® dsDNA quantitation kit to measure the initial DNA mass concentration of the plasmid pIRMM-0073 solution used for processing.

9 CERTIFIED VALUES AND UNCERTAINTY

9.1 CERTIFIED VALUES

The plasmid calibrant is characterised for the number of each of the two specific fragments per plasmid, 356043 and *le1*. The two cloned DNA fragments for which ERM-AD425 is certified, the 356043 event and the *le1*-specific sequences, were found to be identical to the respective PCR targets published with the validated method for quantification of the soybean 356043 event by the EURL-GMFF [11] and certified to be present as one single copy per pIRMM-0073 plasmid each. Based on the sequence and purity assessment, the theoretical number ratio between the two targets is equal to 1.

9.2 UNCERTAINTY EVALUATION

Based on the sequence analyses, it can be concluded that each of the DNA fragments, the 356043 event and *le1*, is present as a single copy per pIRMM-0073 plasmid. Three independent laboratory analyses provided exactly the same sequences which were also the expected sequences as published in the NIH genetic sequence database (GenBank®). The DNA sequencing performed by forward and backward sequencing (on both strands) generated the correct sequence for the 3101 bp analysed. The uncertainty on the sequencing under those conditions can be considered as negligible. The estimated error probability of the sequence identification of each fragment is smaller than 0.000 2 %.

10 METROLOGICAL TRACEABILITY

The certified value is expressed as a number of DNA fragments per plasmid. ERM-AD425 is certified for the number of 99 bp fragments of 5' insert-plant specific (356043 event) DNA and the number of 74 bp *le1* DNA fragments in the plasmid pIRMM-0073. This number is determined on the basis of the full sequence of the plasmid pIRMM-0073 and is traceable to the SI.

The purity of the preparation and the number of each cloned fragment was confirmed by endonuclease restriction. The use of identical vectors to construct pIRMM-0073 originating from single colonies ensures the presence of only one type of plasmid bearing one copy of the 356043 event and the *le1* DNA fragments each. The sequence analysis confirmed the presence of only one type of plasmid in the pIRMM-0073 preparation. Furthermore, the purity of ERM-AD425 was confirmed by restriction analysis, end-point PCR followed by agarose gel electrophoresis.

End-point PCR followed by agarose gel electrophoresis (data not shown) as well as digital PCR were used to further investigate the purity of ERM-AD425. No evidence for contamination with respect to the certified properties was found. However, the ability of the methods applied in this study to investigate the purity of the material was limited and can only ensure a purity of at least 90 %.

11 COMMUTABILITY

The two calibrants tested during the suitability study, pDNA ERM-AD427 and gDNA$_\ell$ extracted from plant leaves, led to significant differences in the measured copy number ratio values of ERM-BF425c (Table 3). Commutability [16] problems should be considered for the copy number ratio measurements of DNA extracted from food and feed samples. It could not be proven during the suitability study that one of the calibrants (pDNA/gDNA$_\ell$) behaves more similar to the gDNA$_s$ extracted from seed powder.

However, based on the traceability chain described above, as well as on additional practical reasons, which include full sequence characterisation, reproduction of additional batches of calibrant and availability, the pDNA ERM-AD425 is selected as the calibrant of choice to be used for the calibration of the event-specific method for the quantification of the 356043 event. The user should be therefore aware, that values measured with the 356043 event-specific and *le1* real-time PCR methods and calibrant ERM-AD425 are reproducible, however, values obtained with this measurement system may not be close to a true value.

12 INSTRUCTIONS FOR USE

12.1 INTENDED USE

The ERM-AD425 is intended to be used as calibrant exclusively with the method for quantification of the soybean 356043 event validated by the EURL-GMFF [11]. This analytical method was validated by the EURL-GMFF for quantification of the 356043 event. The ERM-AD425 can therefore also be used for quantification of the 356043 event present in food and feed products containing this event.

When using ERM-AD425 for the calibration of real-time PCR for the quantification of the soybean 356043 event, the value estimated from the real-time PCR measurement and its related uncertainty should be taken into consideration.

12.2 HANDLING

The plasmid tube should be opened and handled under a laminar flow to reduce the risk of contamination. ERM-AD425 has a number concentration of approximately 2×10^6 cp/µL of the pIRMM-0073 plasmid calibrant. The plasmid solution has to be completely thawed and well mixed prior use. Dilution series should always be prepared freshly on the day of use. $T_1E_{0.01}$ buffer should be used as plasmid dilution buffer. A proposed example of dilution series is presented in ANNEX 2.

12.3 TRANSPORT AND STORAGE

ERM-AD425 shall be dispatched with cooling elements and has to be kept at 4 °C or lower upon arrival. However, for long-term storage, keeping the material at -20 °C is recommended. The serial dilutions of the plasmid should be prepared freshly prior the real-time PCR measurement.

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ANNEX 1 Primer and probe sequences used for the quantification of the 356043 event-specific real-time PCR

	Sequence (5' to 3')	**Reference**
DP-356-f1 (P-0706)	5'-gTCgAATAggCTAggTTTACgAAAAA-3'	[11]
Dp-356-r1 (P-0707)	5'-TTTgATATTCTTggAgTAgACgAgAgTgT-3'	[11]
Dp356-probe (P-0708)	5'-6 VIC-CTCTAgAgATCCgTCAACATggTggAgCAC-TAMRA-3'	[11]
P-0712	5'-ACCCCAAAACCCTCgTCTCTT-3'	this report
P-0713	5'-AgCgACgACTTgATCACCAgA-3'	this report
Lec F (P-0703)	5'-CCAgCTTCgCCgCTTCCTTC-3'	[11]
Lec R (P-0704)	5'-gAAggCAAgCCCATCTgC AAg CC-3'	[11]
Lec P (P-0705)	5'-6 FAM-CTTCACCTTCTATgCCCCTgACAC-TAMRA-3'	[11]

ANNEX 2 Example for the preparation of calibration curves for the endogenous and transgenic targets

An example for the preparation of calibration curves for the endogenous and transgenic targets is given in Table 4. Each ERM-AD425 calibrant is sufficient to prepare 10 calibration curves for each target (see also ERM Application Note 5 [17]).

ERM-AD425 can also be linearised using *Hind*III or *Pst*I restriction endonucleases (single restriction site within pIRMM-0073) which keep the two cloned targets present on pIRMM-0073 intact. Restriction with *Hind*III will keep the *356043* and *le1* fragments next to each other, whereas *Pst*I will leave the cloned targets at both extremities of the pIRMM-0073.

Table 4: Preparation of calibration curves for the endogenous and transgenic targets

Approximate starting number concentration [cp/µL]	Approximate resulting number concentration [cp/µL]	PCR Target [1)]	PCR Target [2)]	Dilution factor	DNA [µL]	Plasmid dilution buffer [µL]
2 000 000	500 000			4	50	150
500 000	100 000	E		5	50	200
100 000	20 000	E		5	50	200
20 000	10 000		T	2	100	100
10 000	2 000	E	T	5	50	200
2 000	1 000	E		2	100	100
1 000	200	E	T	5	50	200
200	20		T	10	50	450
20	5		T	4	50	150

[1)] "E" refers to the number concentrations of the pDNA calibrant that will be used in real-time PCR for the detection of the endogenous target (namely 100 000 cp/µL, 20 000 cp/µL, 2 000 cp/µL, 1 000 cp/µL and 200 cp/µL).
[2)] "T" refers to the number concentrations of the pDNA calibrant that will be used in real-time PCR for the detection of the transgenic target (namely 10 000 cp/µL, 2 000 cp/µL, 200 cp/µL, 20 cp/µL and 5 cp/µL).

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Abstract

This report describes the preparation, characterisation, stability and suitability studies of the certified reference material (CRM) ERM®-AD425 which contains a plasmid (pIRMM-0073) carrying a defined desoxyribonucleic acid (DNA) fragment specific for a genetic modification present in *Glycine max* (L.) Merill line 356043 as well as defined DNA fragment specific for the *Glycine max* taxon.

The CRM was processed in 2007 and certified in 2010 by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE. The CRM is available in plastic tubes containing 500 µL of the plasmid DNA in 1 mmol/L Tris, 0.01 mmol/L EDTA pH 8.0 buffer ($T_1E_{0.01}$) and 1 ng/µl ColE1 plasmid used as background DNA. Each tube contains approximately 10^9 copies of the ERM-AD425 plasmid which correspond approximately to 3.3 ng of DNA.

The plasmid contains a 99 bp fragment of the region that spans the 5' insertion locus of the event 356043 and a 259 bp fragment of the lectin gene (*le1*) specific for the soybean taxon. Both inserted fragments originated from DNA extracted from soybean 356043 seeds supplied by Pioneer Hi-Bred International, Inc. (Johnston, IA, USA).

The certified values are the numbers of cloned DNA fragments for the 356043 and *le1* PCR targets per plasmid.

The recommended minimum sample intake is 50 µL to perform a dilution series. A minimum sample intake of 5 µL was used per real-time Polymerase Chain Reaction (PCR) assay.

The intended use of this CRM is for calibration of the event-specific method for the quantification of the 356043 event validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), "Event-specific method for the quantitation of soybean line DP-356Ø43-1 using real-time PCR", accessible on the homepage of the EURL-GMFF (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm) published on 11/02/2009.

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